Visualizing NO in live cells by confocal laser scanning microscopy

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Received October 20, 2011; accepted November 28, 2011; posted online April 27, 2012

Determination of NO concentration in live cells is essential to evaluate its related cellular functions. In this letter, the concentration of NO in HeLa cells and rat dorsal root ganglion (DRG) neurons are studied by confocal laser scanning microscopy using DAF-2 DA as a fluorescence probe. The results show the fluorescence intensity of NO in HeLa cells is higher than that in DRG neurons, which indicats that the former exhibits higher NO concentration. Furthermore, the experimental conditions for low photobleaching and phototoxicity are optimized.

OCIS codes: 170.0170, 170.1530, 170.1790.
doi: 10.3788/COL201210.S11701.

NO is a gas molecule biosynthesized endogenously by nitric oxide synthase (NOS)[1−3]. It plays vital roles in a wide range of physiological and pathological processes[4,5]. The function of NO is dependent on its concentration in cells. For example, low level concentration of NO can induce proliferation, while high level concentration can lead to apoptotic response[6,7]. Determination of NO concentration in live cells is essential to evaluate its related cellular function.

Due to the short lifetime of NO, it is a challenge to measure directly NO concentration in live cells until the use of NO fluorescent probes. By Combining of confocal laser scanning microscopy[8,9] and NO fluorescence probe, it is possible to detect directly NO in live cells with fine temporal and spatial resolution. Smooth muscle cells, hippocampal neurons, and mast cells are the three main type cells that have been involved in NO fluorescence imaging because that these cells have the typical NOSs, which are endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS), respectively. For example, Kojima *et al.* detected NO fluorescence in rat aortic smooth muscle cells[10]. Zheng *et al.* measured NO fluorescence in cultured hippocampal neurons by confocal laser scanning microscopy[11]. Besides these, there are other kinds of cells that have been used to study the functions of NO in different type of cells. For instance, Vatsa *et al.* measured NO fluorescence in bone cells after mechanical stimulation and found that mechanical stimulation could increase NO production[12]. Lahdenranta *et al.* imaged NO in lymphatic endothelial cells and found NO donor could induce proliferation or survival of lymphatic endothelial cells in dose-dependent manner[13]. However, to the best of our knowledge, few reports have focused on the concentration of NO in HeLa cells and rat dorsal root ganglion (DRG) neurons.

In this letter, the concentration of NO in HeLa cells and DRG neurons were studied by confocal laser scanning microscopy using 4, 5-diaminofluorescein diacetate (DAF-2 DA) as a fluorescence probe. In addition, the experimental conditions were optimized for minimization of light dose during imaging procedure to reduce phototoxicity and photobleaching[14,15].

The human cervical carcinoma cell line HeLa was obtained from college of life science, Fujian Normal University. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, HyClone) supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin-streptomycin, in a humidified incubator with 5% CO_2 at 37 °C. The cells were digested with 0.125% trypsin (Invitrogen) every 3 days and then subcultured into 100-mm culture dish[16].

DRGs were obtained from 8- to 12-week-old Sprague-Dawley rats. The dissected DRGs were incubated in 0.2% collagenase (Class II, Invitrogen) for 20 min at 37 °C and the DRGs digestion were terminated with 10% fetal bovine serum. The remaining tissue were maintained in medium containing 96% Neurobasal, 2% B27, 1% 10 000 units/ml penicillin-streptomycin, and 1% glutamine and triturated to dissociate neurons. The dissociated neurons were plated onto sterile cell culture dish coated with 20 μg/ml poly-D-lysine (Sigma) and fed every 2 days with fresh culture medium[17−19].

HeLa cells and DRG neurons were loaded with 1 μM DAF-2 DA for 45 min in a humidified incubator with 5% CO_2 at 37 °C. The cells were loaded with other concentrations of DAF-2 DA to verify the effect of the fluorescent probe concentration on the fluorescence intensity. After washing 3 times with phosphate buffered saline (PBS), the cells were incubated in PBS and imaged with a Zeiss LSM 510 invert confocal microscope. The main parameters of the confocal microscope were set as 20× (NA=0.4) objective, 1 airy units pinhole, and 750 detection gain. The excitation wavelength and detection wavelength range were set as 488 and 505–560 nm, respectively according to the characteristics of DAF-2 DA[20−22]. The fluorescence intensities were obtained

from cells in one z section, which had the maximum fluorescence, using ImageJ software. The resolution of z section was 6.7 μm when the pinhole and objective magnification were set as 1 airy unit and 20, respectively. More than 15 cells were calculated to get the averaged fluorescence intensities. The fluorescence intensity was chosen as a parameter that presents the ability of reducing photodamage and photobleaching.

The fluorescence probe, DAF-2 DA, could permeate into cells and be transformed quickly into DAF-2 after being hydrolyzed by esterase. DAF-2 reacts with NO to produce DAF-2T, which is highly efficient in fluorescence yield. Figure 1 shows the fluorescence intensity of HeLa cells loaded with 1 μM DAF-2 DA. After adding NOS inhibitor, L-NMMA, the fluorescence intensity was obviously weaker than that in control group. This phenomenon meant that DAF-2 was not transformed into DAF-2T when the NOS was inhibited. However, the fluorescence intensity significantly increased when NO donor, SNP, was added into the same cells because DAF-2 was transformed into DAF-2T after binding NO molecular. Figure 1 demonstrates that 1 μM DAF-2 DA could be suitable for measurement of NO in HeLa cells.

We measured the fluorescence intensity in DRG neurons and HeLa cells loaded with 1 μM DAF-2 DA. Figure 2(a) shows a typical NO fluorescence of DRG neuron. From Fig. 2(a), we could find that the fluorescence of NO in neurite was lower than that in soma. The NO fluorescence of HeLa cells was showed in Fig. 2(b). And Fig. 2(c) shows that there are significant differences (p <0.01) between DRG neurons and HeLa cells on NO fluorescence intensity. This may indicate that NO plays different function in DRG neurons and HeLa cells.

We measured the fluorescence of HeLa cells loaded with five different concentrations of DAF-2 DA. As shown in Table 1, when the concentration of DAF-2 DA was between 0.01 and 0.1 μM, the fluorescence intensity increased a slightly with the increase of DAF-2 DA concentration. However, when the concentration of DAF-2 DA was among a range of 10–100 μM, the fluorescence intensity nearly did not increase with the raise of fluorescence probe concentration due to signal saturation. Therefore, it is best to measure NO fluorescence with 0.1–10 μM DAF-2 DA. The standard deviation was largest at 1 μM concentration, which indicated that

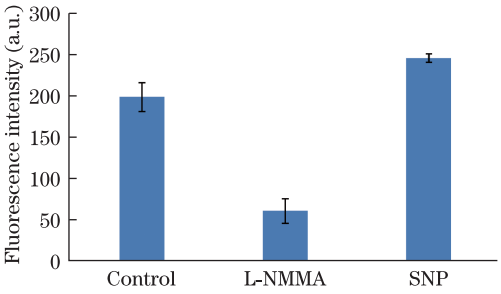

Fig. 1. Fluorescence intensity of HeLa cells loaded with 1 μM DAF-2 DA. The control group is only loaded with DAF-2 DA; the L-NMMA group is preloaded with 1 mM L-NMMA, general NOS inhibitor, 30 min before loaded with DAF-2 DA; the SNP group is that adding 1 mM SNP, NO donor, into the L-NMMA group.

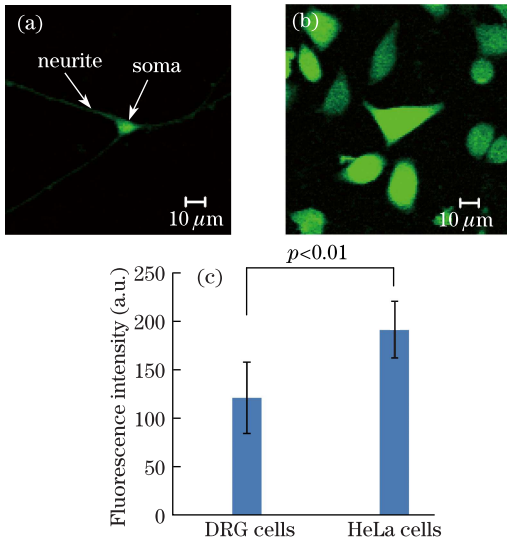

Fig. 2. Fluorescence images of (a) DRG neuron and (b) HeLa cells loaded with 1 μM DAF-2 DA. (c) Fluorescence intensity of DRG neurons and HeLa cells.

Table 1. Effects of the Concentration of DAF-2 DA on NO Fluorescence Intensity

	Concentration of DAF-2 DA (μM)				
	0.01	0.1	1	10	100
Fluorescence Intensity (a.u.)	48.8	63.7	192.6	247.8	249.7
Standard Deviation (a.u.)	6.5	13.7	29.2	5.4	1.9

1 μM DAF-2 DA was the most suitable for measurement of NO in HeLa cells because it could present the diversity of individual cells.

Light dose is a key factor that induces photobleaching and phototoxicity. Therefore, it is of advantage to measure fluorescence signal with low light dose. To verify whether there is linear relationship between light dose and fluorescence intensity, we measure the fluorescence intensity of HeLa cells loaded with 1 μM DAF-2 DA under irradiation by different laser powers. Second-order fit meets better to the raw data than linear fit, as shown in Fig. 3. While at low laser power, the linear fit meets well to the raw data, as shown in the inset. Therefore, it could be possible to compare fluorescence signal in HeLa cells cultured in different dishes using the lowest light dose respectively.

Objective magnification could affect the fluorescence intensity. Our measurement results showed that the fluorescence intensity decreased with the increase of objective magnification. Therefore, it is better to avoid use high magnification if not necessary. However, low magnification means low resolution. In our experimental setup, utilization of 20× (NA=0.4) objective could get satisfactory results both at resolution and fluorescence intensity.

Pinhole could also affect the fluorescence intensity and image quality at the same time. We found that the bigger the pinhole, the stronger the fluorescence intensity.

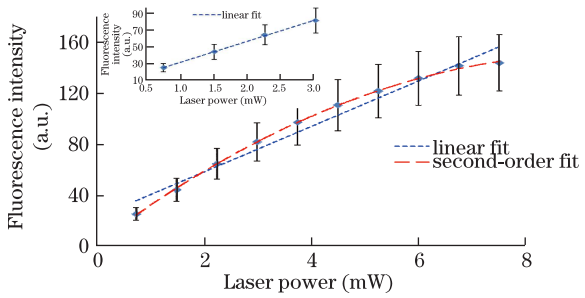

Fig. 3. Non-linear relationship between fluorescence intensity and excitation laser power. The value of excitation laser power presents the output of laser.

However, the image quality will get worse with the pinhole size increase. The optimal pinhole size was 1–1.5 airy units considering the fluorescence intensity and image quality according to our experimental results.

In conclusion, utilizing DAF-2 DA fluorescence probe, NO in DRG neurons and HeLa cells is measured by confocal laser scanning microscopy. The higher fluorescence intensity of HeLa cells than that in DRG neurons indicates that the former exhibits higher concentration of NO. Light dose during imaging procedure is minimized via optimizations of concentration of fluorescence probe, objective magnification, and pinhole. This preliminary study will shed new light on the research about the NO role related to the specific process in live cells.

This work was supported by the National Natural Science Foundation of China (No. 60978071) and the Natural Science Foundation of Fujian Province (No. 2010J01322).

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